A STUDY OF THE DISSOCIATIVE BEHAVIOR OF PSEUDOMONAS AERUGINOSA[1,2]

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Since 1860, when Fordos published his experimental observations concerning the blue-green stain that sometimes appeared on surgical dressings, numerous articles dealing with the organisms commonly assigned to the species *Pseudomonas aeruginosa* have appeared in the literature. Because of its wide and varied range of activity, the definition of this species presents considerable difficulties, and there exists a need for an accurate method of identifying this microorganism. One organism has frequently been described under two names, and specific names have been given to organisms which appear to be merely types or varieties of the species.

These organisms are widely distributed in nature, existing usually as saprophytes, although occasionally giving rise to pathological lesions and generalized infection in man (Lartigau, 1898; Waite, 1908; Fraenkel, 1917; Pons, 1927). Scattered throughout the literature are numerous reports dealing with their pathogenic potentialities for plants (Paine and Branfoot, 1924; Harris, 1940; Naghski, 1941; Elrod and Braun, 1941, 1942; Reid *et al.*, 1942). Perhaps the most outstanding feature of *P. aeruginosa* is the ability to produce a blue-green, chloroform-soluble pigment, pyocyanin. Attempts to determine the nature of the pigment and the circumstances under which the chromogenic properties are manifested have resulted in considerable confusion. Some investigators believe that only one pigment is formed by the different variants and that these variants differ chiefly in their ability to produce ammonia; on the other hand, some maintain that one strain is simultaneously able to produce as many as four distinct pigments. Between these two extremes, we find those who claim that different metabolic pigments may be formed by varying the nutrients of the culture medium.

Jordan (1899) separated the species of *Pseudomonas aeruginosa* into four varieties: (1) pyocyanigenic and fluorescigenic, (2) pyocyanigenic only, (3) fluorescigenic only, and (4) nonchromogenic. In later experiments, Jordan (1903) concluded, from the study of 58 strains of these organisms, that the ability to liquefy gelatin was closely related with the ability to coagulate milk. The organisms capable of liquefying gelatin and coagulating milk were much alike in other characteristics.

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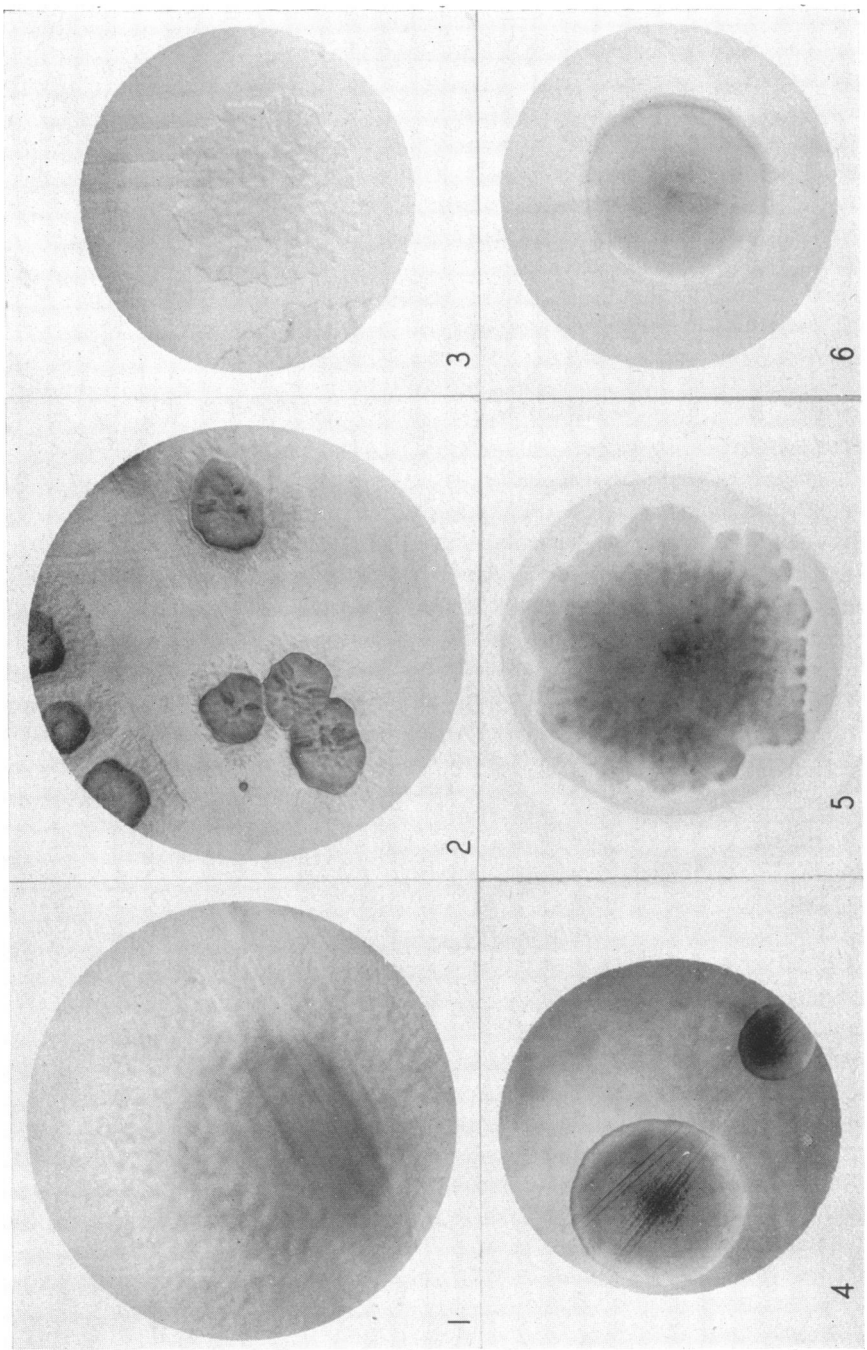
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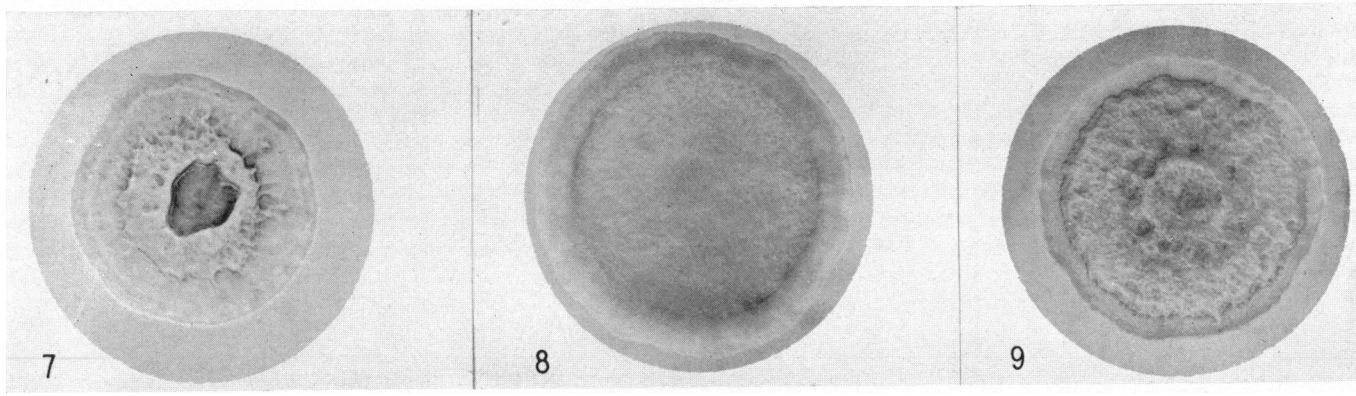

COLONY TYPES AND VARIETIES OF PSEUDOMONAS AERUGINOSA

(Grown on nutrient agar, 3 days old. Magnification × 15)

FIG. 1. Green fluorescent colony of type A.
FIG. 2. Variety of type A green fluorescent colony demonstrating one or more depressed areas.
FIG. 3. Flat, spreading variety of type A green fluorescent colony. The entire colony is similar to the periphery of the type A colony.
FIG. 4. Type B colony responsible for the production of pyocyanin.
FIG. 5. A rough brown pigment-producing colony variety of type B.
FIG. 6. A white colony variety of type B.
FIGS. 7, 8, and 9. Type R colonies responsible for the production of the "Pyo" compounds.

Růžička (1899) separated his cultures of the fluorescent organisms into two groups on the basis of their cultural, morphological, and physiological characteristics, one related to *Pseudomonas aeruginosa* and the other to *Pseudomonas fluorescens-liquefaciens*. Niederkorn (1900) studied 15 strains of the fluorescent microorganisms in an attempt to find better methods for differentiating the various forms. He concluded from these studies that there were only two constant forms, *P. aeruginosa* and *P. fluorescens-liquefaciens*, and that all of the others are varieties of these. Eisenberg (1914) also reported that the fluorescent microorganisms in the group are closely related. He found it difficult to separate them into species and varieties. Tanner (1918) reported that of a group of 100 strains of fluorescent organisms isolated from water, 4 produced endospores, but the rest constituted a homologous group, differences appearing only in regard to liquefaction of gelatin, formation of H_2S, and perhaps fermentation of glycerol.

Meader, Robinson, and Leonard (1925) concluded from their investigation that all typical strains of *P. aeruginosa* produce three water-soluble pigments: (1) fluorescent pigment, (2) pyocyanin, and (3) pyorubin. The pyocyanin and fluorescent pigments act as indicators, but pyorubin remains unaffected in color when treated with acid or alkali. These authors found that the majority of the fluorescent microorganisms which are derived from nature and which are culturally identical with *P. aeruginosa* will produce both pyocyanin and pyorubin on appropriate media and should therefore be classified as *P. aeruginosa*. These authors also reported that agglutination and absorption tests failed to reveal any differences among the various strains tested. Standiford (1937) found that he could not distinguish between *P. aeruginosa* and *P. fluorescens* culturally or serologically; thus he labeled all his strains *P. aeruginosa*. Brooks, Nain, and Rhodes (1925) examined a group of the fluorescent microorganisms associated with plant diseases by cultural, biochemical, and serological methods. By means of the cultural characteristics, the authors were able to arrange their bacteria into three groups: fluorescent, yellow, and white organisms. The biochemical reactions within the first two groups proved to be fairly constant, but preliminary serological examination of these strains revealed important groupings. Elrod and Braun (1942) observed that two cultures of *Phytomonas polycolor* were indistinguishable from certain strains of *P. aeruginosa* by biochemical and serological means. These authors, however, failed to confirm the view of Meader *et al.* that the *P. aeruginosa* group is serologically uniform. Reid and his associates (1942) studied 600 cultures, identified as *Pseudomonas*, and found in addition to serological differences monotrichic and lophotrichic types of flagellation, and differences in ability to grow at 37 C. These authors also described a mucoid phase.

Seleen and Stark (1943) found that the bacteria which produced a green fluorescent pigment were closely related. They divided 199 cultures of these microorganisms into 14 groups on the basis of their ability to grow at 5 C and 42 C, action on milk, liquefaction of gelatin, and reduction of nitrates. Subgroups were determined by the ability of the cultures to produce pyocyanin

and to utilize sucrose, acetic acid, lactic acid, or tartaric acid as the sole source of carbon. Tobie (1945) suggests that the genus *Pseudomonas* should comprise those rod-shaped microorganisms which produce water-soluble phenazine pigments.

ANTIBACTERIAL SUBSTANCES DERIVED FROM PSEUDOMONAS AERUGINOSA

As a result of the early and intensive studies of *P. aeruginosa*, it is not surprising to find that this microorganism was one of the first known to produce a substance antagonistic to other bacteria. Bouchard (1889) observed that the injection of small quantities of a culture of *P. aeruginosa* prevented the development of anthrax in rabbits that had been previously injected with a virulent strain of *Bacillus anthracis*. Woodhead and Wood (1889) found that sterilized cultures of *P. aeruginosa* had the same effect. Other investigators, Charrin and Guignard (1889), Emmerich and Low (1899), von Freudenreich (1888), and Blagovestchensky (1890), called attention to the fact that old cultures and culture filtrates of *P. aeruginosa* were bactericidal for many microorganisms.

The exact nature of this substance aroused a great deal of interest and controversy. Because of certain enzymatic activities of their product, Emmerich and Low named it "pyocyanase." Klimoff (1901) and Dietrich (1902), however, pointed out that the properties of this antibacterial substance were inconsistent with those of an enzyme. Later investigators, Raubitschek and Russ (1909), Ohkubo (1910), and Fukuhara (1911), reported that nearly all the bactericidal material could be extracted with lipid solvents. Thus, they attributed the antibacterial action to lipoids. Gundel and Wagner (1930), Hettche (1932, 1933, 1934), and Hettche and Vogel (1937) concluded that the antibacterial potency of the cellular extract was due to the fatty acid content.

In addition to pyocyanase, *P. aeruginosa* is known to produce another antibacterial substance. This is the blue-green, chloroform-soluble pigment, pyocyanin, the properties of which were investigated by Hettche (1932) and synthesized by Wrede and Strack (1924, 1928, 1929). From the chloroform extracts of *P. aeruginosa* cultures, Schoental (1941) isolated pyocyanin, alpha oxyphenazine, and a pale yellow oil, all possessing antibacterial properties.

Schoental's publication directed this laboratory's attention and interest to a study of the antibiotic materials occurring in cultures and filtrates of *P. aeruginosa*. The resources of the Departments of Bacteriology and Biochemistry were combined in order to investigate this problem in detail. A preliminary paper (Hays *et al.*, 1945) describes in detail the methods and materials used for production of the active materials. Crystalline, active substances have been isolated from crude alcoholic extracts of the cells of cultures five weeks old. These substances have been designated "Pyo" Ib, Ic, II, III, and IV in the chronological order of their isolation.

During the course of this investigation numerous bacteriological problems arose. Foremost among these was the desirability of obtaining a strain capable of producing a large and constant yield of the "Pyo" compounds. An effort was made, therefore, to determine the dissociative activities of *P. aeruginosa*.

Apparently little interest or attention has been directed toward the morphological appearance of the colonies of *P. aeruginosa*, and the few descriptions available are at variance one with another (Tanner, 1918; Ford, 1927; Kramer, 1935; Topley and Wilson, 1937; Park and Williams, 1939; Bergey *et al.*, 1939; Jordan and Burrows, 1942). However, Lartigau (1898), Hadley (1927), and Kramer (1935) called attention to the fact that *P. aeruginosa* is subject to variation under conditions of laboratory cultivation. Hadley observed that many cultures lose their ability to produce the chloroform-soluble, blue-green pigment. When such cultures are plated out, it was found that all of the colonies did not produce the blue-green color, but some merely possessed a yellow fluorescent pigment. Kramer further observed that the colony morphology of *P. aeruginosa* is also subject to variation. It appears fairly certain to us that this variation in pigment production alone accounts for much of the confusion encountered in the literature concerning the identification and classification of this microorganism. It is also possible that, with the change in pigment production, there may be associated changes in other characteristics as well. Therefore, a detailed examination of the various colony forms was undertaken in an attempt to determine the possible range of variability, if any, and also to catalogue those features which may characterize the appearance of the typical *P. aeruginosa* colony.

METHODS

Terminology. When the fluctuation in the generic and species terminology is considered, it seems rather futile to attempt to standardize the nomenclature of the dissociative subdivisions occurring in the species. The term "strain" is satisfactory only when referring to a stable daughter colony possessing characteristics different from that of the parent colony. The term "clone" is an excellent one when referring to the stable progeny of a single cell, and its use should not be abused. A review of the literature reveals a general overlapping in the meaning of all the popular terms, the choice of which is apparently left entirely to the investigator. Therefore, in this paper the term "type" will be used to refer to the cultures derived from selected colonies of the parent culture either by means of artificial selection or by means of dissociative activities. The term "variety" will be used in reference to any visible, external, dissociative tendencies occurring in the colony type. Thus we have genus, species, type, and variety.

Cultures employed. The *Pseudomonas aeruginosa* cultures were obtained directly from infected lesions whenever possible; thus, any changes or dissociations that developed could be accurately evaluated.

P-SLU, a stock strain maintained in our laboratory for the past several years, originally derived from a pathological lesion. A strong pyocyanin producer.

P-CC, isolated from a chronic bladder infection. A mediocre pyocyanin producer when first isolated, but this property was soon lost.

P-B, isolated from an infected burn. A strong pyocyanin former.

P-M, isolated from an abscessed tooth. Produces an abundance of pyocyanin.

P-U, *P-U1*, *P-U2*, *P-U3*, cultures received as *P. aeruginosa*, isolated from urine. These cultures produced an abundance of fluorescin but no pyocyanin.

P-S, isolated from a blood culture of a patient suffering from septicemia. A strong pyocyanin producer.

P-Ky, stock culture from the University of Kentucky. The culture produced no pigment.

P-Ky1, stock culture from the University of Kentucky. A strong producer of pyocyanin.

P-9027, obtained originally from the A.T.C.C. This culture had recently been grown on lettuce leaves.

Throughout this investigation all colony studies were made on plain nutrient agar using the streak plate method. This method was found to be superior to the pour plates, as only surface colonies were desirable and it was more convenient for the handling of a large number of cultures. All incubations were at 37 C. Previous experience indicated that the plates should be incubated for 72 hours to ensure complete development of all colonies and to permit the appearance of any dissociative tendencies which might be present. Shorter periods of incubation may result in immature colonies and thus give rise to false interpretations; if incubated for longer periods, secondary or daughter colonies may develop. A small hand lens mounted on a ring stand was found to be very satisfactory for examining the colonies.

As culture *P-CC* was being employed routinely for the production of the "Pyo" compounds, this culture was used for most of the preliminary studies. When streak plates were made for a comparative study of the colonies, *P-CC* presented such a confusing variety that it appeared on first inspection to be contaminated. The colonies varied from blue-green in color to white, and from small, round, convex, opaque, to large, rough, spreading, flat, translucent forms. The ability of this culture to produce pyocyanin was slight, and this property was soon lost altogether.

With the aid of the lens a representative colony form of each major type present was selected, emulsified in nutrient broth, and restreaked on nutrient agar. These various colony types were followed through numerous generations in this manner until the respective types were 100 per cent reproducible or stable. This procedure was complicated by the fact that various colonies would lose their ability to produce pigment, not only pyocyanin, but fluorescent pigment as well. The latter may not be an actual loss in the true sense of the word, but rather it is possible, by colony selection, to separate from the fluorescent colony type a nonfluorescent variety that is identical in every other respect with the parent.

RESULTS

Colony types. As a result of these studies, it soon became apparent that the culture (*P-CC*) was composed of three basic colony types, which have been, for the lack of better terminology, called types A, B, and R.

Type A colonies appear as circular, smooth or undulate, convex; with trans-

lucent centers; effuse, flat, wavy, transparent periphery; and irregular, lobulated edges. These are the yellow fluorescent pigment producers and the predominant colony type of the culture.

Type B colonies are small, round, convex, translucent, and finely granular with entire edges. This colony type produces the blue-green pigment, pyocyanin. Frequently type B colonies are contained in and completely masked by type A colonies.

Type R colonies were somewhat variable; usually round or slightly irregular and curled, raised, or flat; umbilicate or umbonate; finely or coarsely granular; and at times almost filamentous. Edges might be entire, undulated, lobate, or auriculated. Although inconsistent in their morphological appearance, these variations were not sufficient to warrant new or different types.

Numerous combinations of these types may occur with corresponding colony configurations. It is also possible by appropriate means, such as animal passage, growing in high concentrations of glycerol, or repeated transfers in increasing concentration of homologous antiserum, to convert one colony type into another. Furthermore, each of the three basic colony types is subject to variation. Type A may be forced to throw off an A-depression variety, similar to the type A colony but exhibiting one or more depressed areas in the center. Type B may be forced to throw off a stable B-rough-brown variety, a coarsely granular, convex colony with irregular edges, and producing a diffusible, dark brown, chloroform-insoluble pigment.

The colony morphology of cultures *P-M*, *P-B*, and *P-S* is identical with that of *P-CC*. The urine cultures, *P-U*, *P-U1*, *P-U2*, and *P-U3* exhibited only type A colonies. *P-Ky* was a stable, nonpigmented variety of a type B colony, whereas *P-Ky1* was an unstable rough variety of type A. Culture *P-9027* was made up of stable type A colonies. Culture *P-SLU*, originally employed for the production of the "Pyo" compounds, but the use of which was discontinued when the crude extracts failed to produce the usual yield of active material, was made up of the typical type B colonies, and a large, smooth, spreading B variety. Types A and R were entirely lacking from this culture.

From these observations it seems plausible to assume that cultures derived from naturally occurring human infections are made up of more than one colony type. Whether these types were naturally occurring, or whether they originated from the dissociation of only one type, is debatable. The latter is probably more correct as shown by the fact that the colony types are subject to dissociation.

The derived varieties are reproducible from generation to generation by transferring carefully selected colonies, but they are by no means stable or permanent. The three basic colony types remain stable under ordinary conditions of laboratory cultivation, but dissociation occurs in old cultures. Occasionally permanent types or varieties are thrown off, i.e., a type B colony has been obtained that remains stable with 100 per cent of the colonies producing the blue-green pigment. Mouse passage, for which animal this particular type is highly fatal, resulted only in intensifying the pigment production.

Colonies exhibiting a "lytic" action similar to that described by Hadley (1924) were observed. This lytic action appeared only in the pyocyanin-producing B type colonies. With the aid of a straight needle, portions of these pitted areas were inoculated into young normal colonies and also onto fresh agar plates. This property was found to be nontransferable to the young colonies and reproducibility on fresh agar was extremely uncertain. It seems very unlikely that this action was due to a bacteriophage, although this possibility was by no means extensively investigated. It was also of interest to note that at various times numerous G type colonies would appear. These extremely small colonies could not be explained (crowding was not a factor) nor were they reproducible. These colonies are mentioned only to point out the extreme variations found.

When the colonies are transferred to nutrient broth, growth is more or less typical of the type. The B types are facultative anaerobes which grow uniformly throughout the medium with very little pellicle formation. When the tube is shaken the pellicle breaks up into very small fragments with little tendency to settle to the bottom. Type A, a strict aerobe, forms a heavy pellicle with only slight turbidity throughout the medium. When shaken the pellicle tends to break up into two or more clumps and to settle to the bottom. Type R, also a strict aerobe, grew only on the surface with the formation of a dense, almost mucoid, wrinkled pellicle. When shaken the pellicle forms a gummy, sticky mass which tends to adhere to the sides of the tube or flask just above the surface of the medium, but when shaken free will settle to the bottom. A new pellicle forms rapidly. Although type R produced a somewhat mucoidlike growth in broth and on agar, these organisms, including types A and B, did not produce capsules, nor did they give the slightest indication of encapsulation under any conditions.

Biochemical characteristics of the various colony types. The biochemical characteristics of these microorganisms are likewise extremely variable and few authors seem to agree on their fermentation patterns. The complexity of their extremely active endoenzyme and exoenzyme systems is such that classification of these organisms by this means alone is impossible. All varieties of *P. aeruginosa* studied will grow in a simple synthetic medium and will utilize glycerol or glucose as the sole source of carbon. In addition to a hemolytic enzyme, an active and independent proteolytic enzyme is produced; indole is not formed from tryptophane; and nitrates may or may not be reduced to nitrites. All types and varieties, with one exception, produced acid without gas from glucose and glycerol, whereas others produced acid without gas from glucose, glycerol, xylose, and galactose. One variety of culture *P-SLU* did not ferment any of the test substances.

An attempt was made to correlate fermentation pattern with colony types and varieties. The results recorded in table 1 indicate that while a slight correlation may be possible, it is by no means complete. It should be emphasized that these biochemical reactions are somewhat difficult to repeat. All of the culture characteristics listed in the table represent the average of a number of repeated tests employing young cultures. This lack of uniformity leaves one

TABLE 1

Biochemical reactions

CULTURE	VARIETY OR TYPE	GELATIN	LITMUS MILK	GLUCOSE	XYLOSE	GALACTOSE
P-CC	B	L 14 days	C 72 hr	+ 48 hr	+ 72 hr	+ 72 hr
	A	L 24 hr	D 48 hr	+ 24 hr	+ 24 hr	+ 24 hr
	R	L 24 hr	D 48 hr	+ 24 hr	+ 24 hr	+ 24 hr
	B Brown	L 7 days	C 72 hr	+ 48 hr	–	–
	A Dep.	L 24 hr	D 72 hr	+ 24 hr	+ 24 hr	+ 24 hr
P-B	B	L 14 days	D 72 hr	+ 24 hr	+ 24 hr	+ 96 hr
	A	L 24 hr	D 72 hr	+ 24 hr	+ 24 hr	+ 24 hr
	R	L 24 hr	D 48 hr	+ 24 hr	+ 24 hr	+ 24 hr
P-SLU	B	L 14 days	D 7 days	+ 48 hr	–	–
	Large B	– 14 days	– 14 days	–	–	–
P-S	A	L 24 hr	D 48 hr	+ 24 hr	+ 72 hr	+ 72 hr
	B	L 72 hr	D 96 hr	+ 24 hr	+ 48 hr	+ 48 hr
	R	L 24 hr	D 48 hr	+ 24 hr	+ 24 hr	+ 24 hr
P-U	A	L 24 hr	D 96 hr	+ 48 hr	–	–
P-M	B	L 24 hr	D 72 hr	+ 24 hr	+ 24 hr	+ 24 hr
	A	L 24 hr	D 48 hr	+ 24 hr	+ 24 hr	+ 24 hr
	R	L 24 hr	D 48 hr	+ 24 hr	+ 24 hr	+ 24 hr
P-9027	A	L 48 hr	D 72 hr	+ 48 hr	–	–

L = Liquefaction; C = Curd; D = Digestion; + = Acid production; – = No reaction.

in doubt as to a means of identifying this microorganism. It is, therefore, convenient and certainly not subject to very severe criticism to consider those bacteria which are gram-negative, nonsporeforming, possessing 1 to 8 polar

flagella, actively proteolytic and hemolytic, producing a blue-green or fluorescent pigment (whether or not chloroform-soluble), and producing acid without gas from glucose, as belonging to the species *Pseudomonas aeruginosa.*

Antigenic pattern. Meader, Robinson, and Leonard (1925) reported variations in the agglutinin content of different antisera, but absorption with any of their cultures resulted in complete reduction of both homologous and heterologous reaction. They concluded, therefore, that the group is serologically uniform. Aoki (1926) concluded from his experiments that the microorganisms are antigenically dissimilar. Harris (1940), Naghski (1941), and Reid *et al.* (1942) reported "M" and "S" phases of the organism. The "M" or encapsulated phase was naturally occurring, but the "S" phase was induced by cultivation in homologous immune sera. Their agglutination and cross-agglutination tests indicated that there was a close antigenic relationship of the organisms in the Dawson "M" phase, but that old stock cultures were not so antigenically similar to freshly isolated cultures. The microorganisms in the "S" phase indicated a homologous serological relationship. Harris reported three distinct serological types in the Dawson "M" phase. Elrod and Braun (1942) and Munoz, Scherago, and Weaver (1945) found the group to be serologically heterologous.

In this investigation, antisera were prepared against each of the basic colony types and varieties of culture *P-CC*, and of cultures *P-SLU*, *P-Ky*, *P-U*, and *P-9027*. Both formalin- and alcohol-treated cells were prepared. Formalin is known to preserve and even increase to some extent the agglutinability of motile bacteria, and alcohol destroys the agglutinability of the H antigen; but the O antigen retains its antigenic properties. The antisera were prepared by suspending the growth of 18-hour agar cultures in formalinized saline and injecting rabbits every other day by the intravenous route with increasing dosages of the cell suspension. The amount of antigen per injection was found to be of secondary importance, but it was desirable to obtain the maximum antibody production in the minimum length of time to prevent broadening of the antigenic pattern. Dosages starting with 0.25 ml and doubling with each subsequent injection until 2 ml were reached were found to give satisfactory titers as shown by trial bleedings from the ear artery. Following this series of injections, living organisms were given in the same manner with a resulting slight increase in titer. Subcutaneous injections of the antigen are satisfactory, but the time factor is increased and the antibody titers are, on the whole, slightly lower.

Because of the variability of these colony types, it was desirable to prepare sufficient quantities of the antigens to last throughout the investigation. Agglutination, cross-agglutination, and agglutinin-absorption tests were then run in an attempt to correlate the various colony types. All H agglutinations were incubated in the water bath at 55 C for 2 hours, although agglutination was usually complete in less than 30 minutes. The O agglutinations were incubated at 55 C for 8 hours and overnight at icebox temperature. The method of adding the cell suspension to the decreasing concentrations of antisera was entirely arbitrary. The results obtained by adding one drop of a heavy cell suspension to each tube were identical with those observed when one ml of a thin cell sus-

TABLE 2

"O" type agglutination

AGGLUTINATING CELLS	ANTISERA					
	A-*P-CC*	B-*P-CC*	R-*P-CC*	A-*P-U*	A-*P-9027*	*P-SLU*
A-*P-CC*	1,280*	320	1,280	1,280	640	640
B-*P-CC*	1,280	320	1,280	640	1,280	1,280
R-*P-CC*	1,280	320	1,280	1,280	1,280	640
A-*P-U*	1,280	160	1,280	1,280	640	640
A-*P-9027*	640	320	640	320	1,280	320
P-SLU	320	320	320	320	160	1,280
A-*P-U1*	1,280	160	1,280	1,280	320	640
A-*P-U2*	1,280	160	1,280	1,280	320	640
A-*P-U3*	1,280	160	1,280	1,280	320	640
A-*P-B*	1,280	320	1,280	1,280	640	640
B-*P-B*	640	320	640	1,280	640	1,280
R-*P-B*	1,280	320	1,280	1,280	1,280	640
A-*P-M*	1,280	160	640	640	1,280	640
B-*P-M*	1,280	160	640	1,280	640	1,280
A-*P-S*	1,280	160	640	640	1,280	640
B-*P-S*	640	160	640	640	640	1,280

* Figures represent the highest dilution showing 4 plus agglutination.

TABLE 3

"H" agglutination

FORMALIN-TREATED CELLS	ANTISERA								
	A-*P-CC*	B-*P-CC*	R-*P-CC*	A-*P-9027*	*P-Ky*	*P-SLU*	A-Dep. *P-CC*	B-Br. *P-CC*	A-*P-U*
A-*P-CC*	>10,240	1,280	>10,240	2,560	320	320	>10,240	5,120	>10,240
B-*P-CC*	5,120	5,120	>10,240	320	>10,240	>10,240	>10,240	>10,240	1,280
R-*P-CC*	>10,240	1,280	>10,240	640	>10,240	2,560	>10,240	>10,240	2,560
A-*P-9027*	2,560	80	320	>10,240	40	1,280	80	80	640
P-Ky	20	40	<20	<10	>10,240	<20	<20	<20	<20
P-SLU	640	1,280	640	320	160	>10,240	160	320	320
A-Dep. *P-CC*	>10,240	1,280	>10,240	640	>10,240	160	>10,240	5,120	>10,240
B-Br. *P-CC*	160	5,120	2,560	320	>10,240	320	>10,240	>10,240	320
A-*P-M*	>10,240	640	5,120	640	>10,240	320	>10,240	2,560	>10,240
B-*P-M*	2,560	5,120	5,120	320	>10,240	>10,240	5,120	>10,240	1,280
R-*P-M*	>10,240	1,280	>10,240	640	>10,240	1,280	5,120	5,120	1,280
A-*P-B*	>10,240	640	5,120	2,560	>10,240	160	>10,240	1,280	2,560
B-*P-B*	2,560	5,120	5,120	320	>10,240	>10,240	5,120	5,120	1,280
R-*P-B*	>10,240	5,120	>10,240	2,560	>10,240	160	>10,240	1,280	5,120
A-*P-U*	>10,240	640	5,120	1,280	>10,240	320	>10,240	1,280	>10,240
A-*P-S*	>10,240	640	5,120	1,280	>10,240	320	>10,420	2,560	>10,240
B-*P-S*	2,560	5,120	2,560	160	>10,240	1,280	5,120	>10,240	1,280

pension was added. It is to be observed in tables 2 and 3 that specific O and H agglutinins were demonstrable in the blood serum of rabbits inoculated

with formalinized cell suspensions. The flagellar agglutinins may be absorbed out, leaving the somatic agglutinins unaffected (table 4). It is to be further observed that reciprocal agglutinin absorption showed that the O types are serologically identical regardless of the colony types. If one should consider only the somatic agglutinogens and their respective agglutinins, the results would indicate that the *P. aeruginosa* group is serologically homologous. Differences are apparent only when testing for the flagellar agglutinogens. Even then, as is evident in table 3, the group appears more or less homologous. Culture *P-Ky* was entirely out of line with the other types, there being no correlation whatsoever. However, it would seem from the agglutinin content of its antiserum that the flagella or cell contains complete agglutinogens but that these were present in such small quantities that they were not demonstrable under the conditions of the test. Neither could culture *P-Ky1* be correlated with the other colony types. This culture is not included in the tables.

TABLE 4

"O" agglutination

ALCOHOL-TREATED CELLS	B-*P-CC* ANTISERUM ABSORBED WITH "H" CELLS OF				A-*P-U* ANTISERUM ABSORBED WITH "H" CELLS OF			
	A-*P-CC*	B-*P-CC*	R-*P-CC*	A-*P-U*	A-*P-CC*	B-*P-CC*	R-*P-CC*	A-*P-U*
A-*P-CC*	320	320	320	320	1,280	1,280	1,280	1,280
B-*P-CC*	320	320	320	320	640	640	640	640
R-*P-CC*	320	320	320	320	1,280	1,280	1,280	1,280
A-*P-U*	160	160	160	160	1,280	1,280	1,280	1,280

The absorption of A-*P-CC* and R-*P-CC* antisera with the "H" cells had no effect on the agglutinability of the alcohol-treated cells. Both antisera agglutinated to titer, i.e., 1,280.

A summary of the results of the flagellar agglutinin-absorption tests is presented in table 5. It is evident that the three colony types, A, B, and R, are serologically heterologous when tested in this manner. The majority of these types are not pure, that is, they contain some agglutinogens of heterologous types. This is particularly true when the colony types have been derived from the same culture. However, it is possible that pure types or varieties could be developed by continuous colony selection and transfer, or by single cell isolation.

"Pyo" production. As the original purpose of this investigation was partly to obtain a culture of *P. aeruginosa* capable of producing greater and more constant yields of the "Pyo" compounds, each experimentally developed colony type was investigated for its ability to produce these antibiotic materials. As *P-CC* was known to be a satisfactory producer of "Pyo," each of the colony types from this culture was inoculated into routine culture carboys. The types used were known to remain stable for at least 2 weeks when grown in a liquid medium in the test tube. The growth of each type was carefully followed by streaking samples on nutrient agar at weekly intervals. It was always desirable to know the type of organism contained in the pellicle as well as the type growing

throughout the medium. Type B remained stable during the 5-week period. Because it was a facultative anaerobe, growth was abundant throughout the

TABLE 5

Agglutinin-absorption reaction

AGGLUTINATING CELLS	A-*P-CC* ANTISERUM ABSORBED WITH "H" CELLS OF					
	A-*P-CC*	A-*P-U*	A-*P-B*	B-*P-CC*	B-*P-B*	R-*P-CC*
A-*P-CC*	<40	<40	<40	2,560	10,240	320
A-*PU*	<40	<40	<40	10,240	10,240	1,280
A-*PB*	<40	<40	<40	5,120	1,280	1,280
A-*P-M*	<40	<40	<40	10,240	10,240	2,560
A-*P-S*	<40	<40	<40	5,120	10,240	2,560
A-*P-9027*	<40	<40	<40	1,280	1,280	1,280
B-*PCC*	<40	80	<40	<40	<40	640
B-*P-B*	<40	40	<40	<40	<40	640
B-*P-S*	<40	80	<40	<40	<40	1,280
R-*P-CC*	<40	160	80	640	5,120	<40
	B-*P-CC* ANTISERUM ABSORBED WITH "H" CELLS OF					
	B-*P-CC*	B-*P-M*	B-*P-B*	A-*P-CC*	A-*P-U*	R-*P-CC*
B-*P-CC*	<40	<40	<40	640	2,560	320
B-*P-M*	<40	<40	<40	1,280	1,280	1,280
B-*P-B*	<40	<40	<40	2,560	1,280	640
B-*PS*	<40	<40	<40	2,560	1,280	1,280
B-Brown *P-CC*	<40	<40	<40	640	1,280	320
A-*P-CC*	<40	<40	<40	<40	<40	640
A-*P-U*	<40	<40	<40	<40	<40	1,280
A-*P-B*	<40	<40	<40	<40	<40	1,280
R-*P-CC*	<40	<40	<40	320	1,280	<40
	R-*P-CC* ANTISERUM ABSORBED WITH "H" CELLS OF					
	R-*P-CC*	R-*P-M*	R-*P-S*	B-*P-CC*	A-*P-CC*	A-*P-M*
R-*P-CC*	<40	<40	<40	1,280	640	1,280
R-*P-M*	<40	<40	<40	5,120	1,280	1,280
R-*P-S*	<40	<40	<40	5,120	1,280	2,560
R-Spreading *P-CC*	<40	<40	<40	1,280	320	1,280
B-*P-CC*	<40	<40	<40	<40	2,560	5,120
B-*P-M*	<40	<40	<40	<40	5,120	1,280
A-*P-CC*	<40	<40	<40	640	<40	<40
A-*P-M*	<40	<40	<40	1,280	<40	<40
A-Dep-*P-CC*	<40	<40	<40	640	160	320

medium with only scant pellicle formation. When the carboy was shaken, the pellicle broke up into numerous pieces and had little tendency to settle to the bottom. Extraction of these 5-week-old cells yielded approximately one-fourth

of a satisfactory antibacterial titer. Type A did not remain stable during the 5-week period; both B and R types were thrown off. The antibacterial titer was found to be directly related to the number of R type organisms present in the pellicle. The carboy inoculated with type R produced a very satisfactory yield of "Pyo." This fact was confirmed by inoculating routine batches of carboys (24 carboys per batch). The growth was typical of the R type with a heavy, wrinkled, slimy pellicle and with scant growth throughout the medium. Similar experiments employing types from other cultures conclusively proved that type R was responsible for the production of "Pyo."

DISCUSSION OF THE RESULTS

Cultures of *P. aeruginosa* isolated from infections in man have been shown to vary considerably in the morphological appearance of their colonies. By the transfer of selected colonies it has been possible to demonstrate that these numerous variants are derived from at least three basic colony types. For a lack of better terminology these types have been designated A, B, and R. By a variety of means it has also been possible to demonstrate that any one of these basic types may be transformed into another type. There is also sufficient evidence to support the view that the dissociation of these organisms may occur *in vivo* as well as under various environmental conditions *in vitro*. It is of interest to note that the colonies of type A described above are indistinguishable from those of *P. fluorescens* as described elsewhere, and it is quite possible that they are identical. This same theory might well account for many of the recently described species occurring in the genus *Phytomonas* that are serologically indistinguishable from *P. aeruginosa*. In view of the experimental evidence presented it seems reasonable to assume that a great deal of the present confusion encountered in the literature concerning this species can be explained adequately by the dissociative behavior of the various cultures studied.

The morphological appearance of the typical *P. aeruginosa* colony is subject to considerable discussion. If the ability of a culture to produce pyocyanin continues to be an essential criterion for the identification of *P. aeruginosa*, then we must consider the type B colony described above as typical of the species. However, the fluorescent colonies of type A were in general more numerous, and it is the opinion of this investigator that the experimental evidence points to this colony type as being typical of the species. On the other hand, the typical colony morphology may be of only minor importance when one considers the dissociative ability of the species and the tendency to lose its ability to produce the blue-green pigment, pyocyanin. A comparative examination of stock cultures that have been carried for a number of years in the laboratory will further prove the dissociative behavior of *P. aeruginosa*, not only in the morphological appearance of the colonies but also in their biochemical characteristics as well. The biochemical characteristics of these microorganisms are entirely unreliable and, with the exception of the reactions in gelatin, litmus milk, and glucose, are apparently of little importance. The organisms of types A and R were, on the whole, more versatile than those of type B.

With regard to the serological pattern of *P. aeruginosa* it is evident that this species is serologically heterologous if we consider that the culture contains a variety of colony types. However, similar colony types obtained from various cultures proved to be serologically identical. Therefore, it is reasonable to assume that the various conflicting results which have been reported are due to the heterologous colony types employed in the various studies. One also finds that the majority of the authors are not specific in stating whether the O or H type of agglutination was observed; thus the type can only be assumed from the agglutinating titers which they have recorded. If this be true, then some of the investigators were no doubt studying the somatic antigens and others were investigating the flagellar antigens. Naturally, the two sets of results appear contradictory, but, in view of the evidence presented above, both may actually be correct. The somatic antigens are serologically homologous, but agglutinin-absorption tests show a definite heterologous relationship existing between the flagellar antigens of the three basic colony types. The flagellar agglutinogens of identical colony types, however, are serologically homologous.

The "M" phase of *P. aeruginosa*, described by Reid and his associates, was not observed in this investigation. Colonies of the R type may be described as somewhat mucoidlike, but the organisms were entirely devoid of capsules.

SUMMARY

The results of a study of the dissociation of *Pseudomonas aeruginosa* have been reported. By appropriate means it was possible to demonstrate that colonies derived from cultures of *Pseudomonas aeruginosa* isolated from human infections vary considerably in their morphological appearance. These colony variations were numerous and were shown to be derived from at least three basic colony types designated herein as types A, B, and R.

The patterns of fermentative and proteolytic activities of these colony types were extremely variable and apparently would be of little value as a means of classification.

Agglutination reactions of the various cultures of *P. aeruginosa* indicated that the somatic antigens are homologous, but agglutinin-absorption tests show a definite heterologous relationship existing between the flagellar antigens of the three basic colony types.

The types of *P. aeruginosa* responsible for the production of the "Pyo" compounds and pyocyanin have been identified and described.

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